

# CHEM**BIO**CHEM

## Supporting Information

### **Developing an Antibody–Drug Conjugate Approach to Selective Inhibition of an Extracellular Protein**

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cbic\_201800623\_sm\_miscellaneous\_information.pdf

## **Author Contributions**

*E.L. Conceptualization: Lead; Formal analysis: Lead; Investigation: Lead; Methodology: Lead; Project administration: Lead; Resources: Lead; Validation: Lead; Visualization: Lead; Writing – original draft: Lead; Writing – review & editing: Lead; Joint first author: Lead*

*A.S. Conceptualization: Lead; Formal analysis: Lead; Investigation: Lead; Methodology: Lead; Project administration: Lead; Resources: Lead; Validation: Lead; Visualization: Lead; Writing – original draft: Lead; Writing – review & editing: Lead; Joint first author: Lead*

*H.C. synthesis: Supporting*

*K.G. Writing – original draft: Supporting; synthesis: Supporting*

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*S.P. Investigation: Supporting; Supervision: Supporting*

*D.M. Conceptualization: Supporting; Resources: Supporting; Supervision: Supporting*

*A.M. Conceptualization: Supporting; Resources: Supporting; Supervision: Supporting.*

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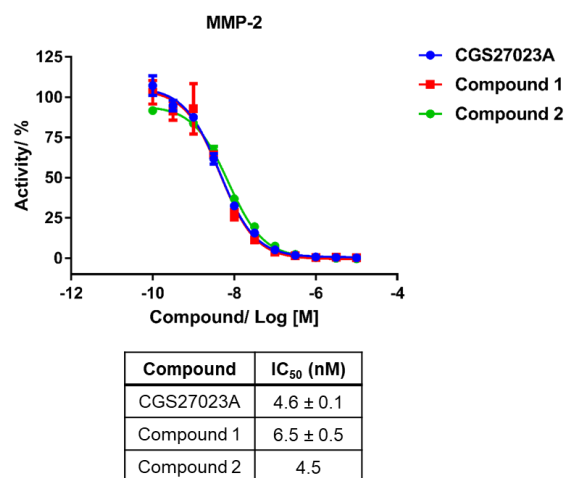
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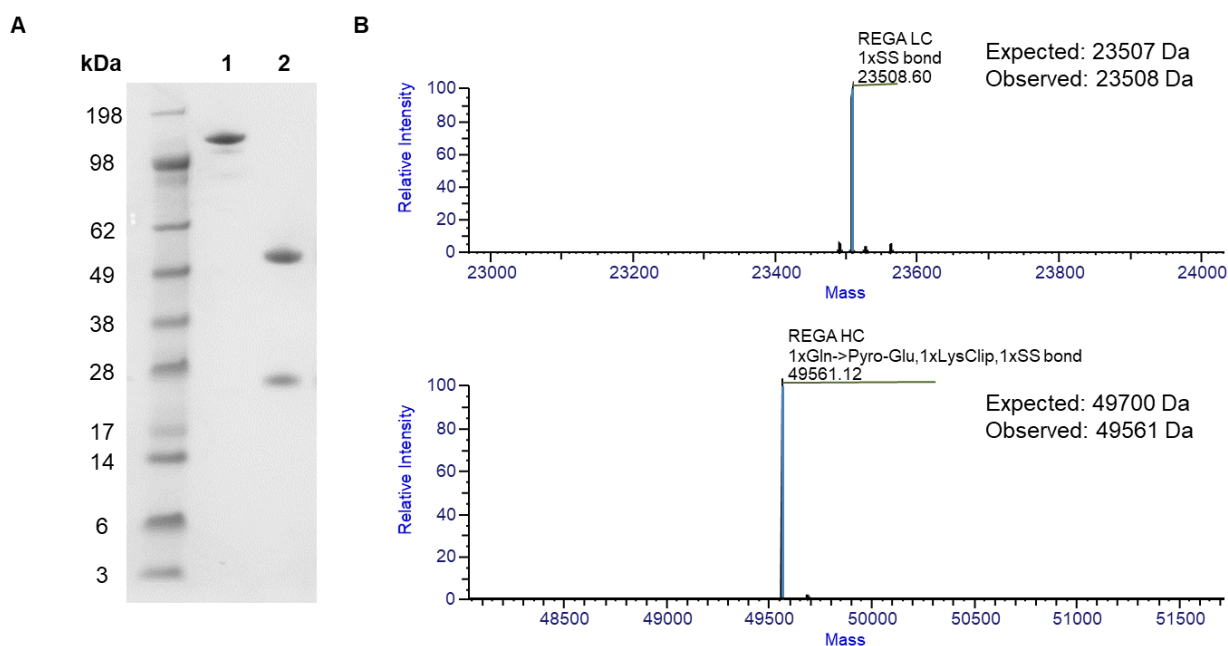
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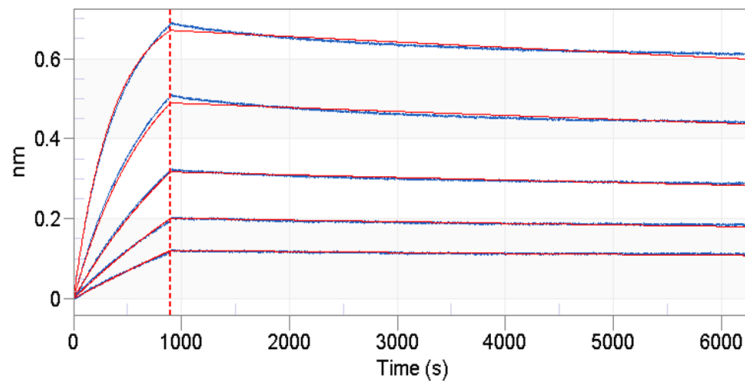
## Supplementary Figures



**Figure S1. Inhibition of human MMP-2 activity by CGS27023A, 1 and 2 in the SensoLyte fluorometric assay.** Data were normalized to the MMP-2 alone control and are expressed as means ± SEM for three independent experiments.

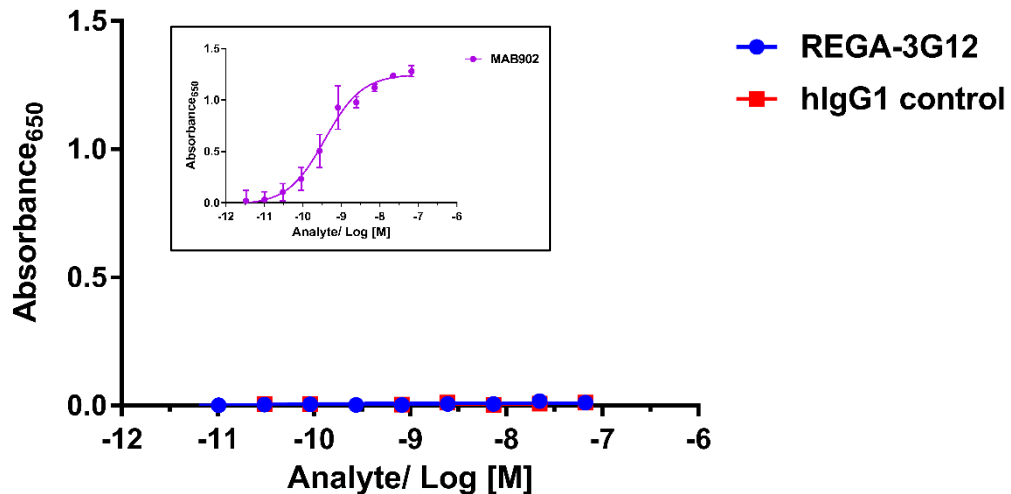


**Figure S2. Characterization of REGA-3G12** A) SDS-PAGE analysis of REGA-3G12. *Lane 1*; REGA-3G12. *Lane 2*; reduced REGA-3G12. B) Mass spectrometry analysis of REGA-3G12 light chain (LC) and deglycosylated heavy chain (HC). Masses matched to sequence using BioPharma Finder software (Thermo Scientific). The N-glycans were removed by incubating with Rapid PNGase F (New England Biolabs) for 1 h at 45 °C, then REGA-3G12 was reduced by incubating with 50 mM DTT at 37 °C for 30 min.

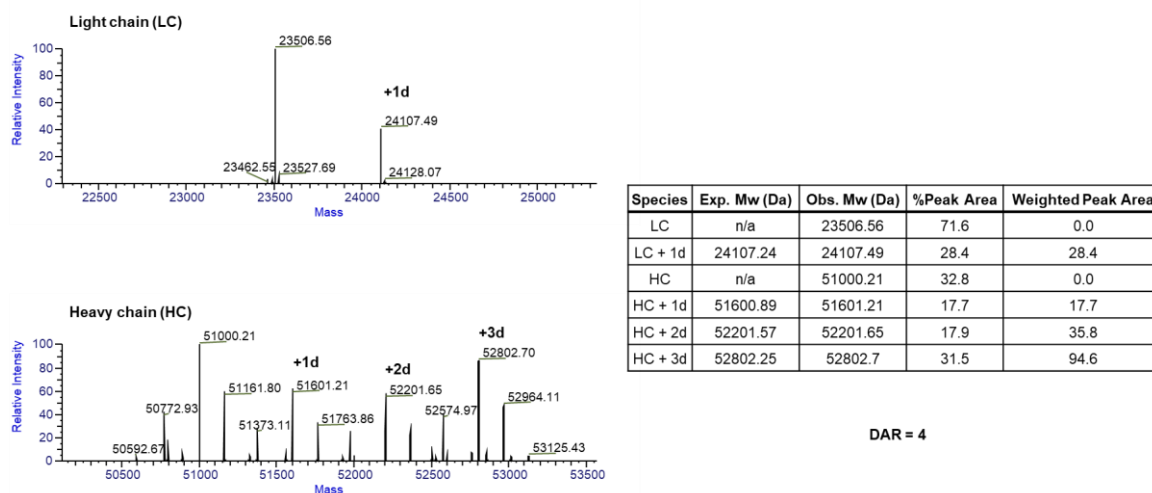


Loading Sample ID	Sample ID	Conc. (nM)	Response	KD (M)	KD Error	kon(1/Ms)	kon Error	kdis(1/s)	kdis Error	Full X^2	Full R^2
REGA-3G12	Hu MMP-9 catalytic domain	12.5	0.685	9.74E-11	<1.0E-12	2.18E+05	2.94E+02	2.12E-05	<1.0E-07	0.723	0.999
REGA-3G12	Hu MMP-9 catalytic domain	6.3	0.503	9.74E-11	<1.0E-12	2.18E+05	2.94E+02	2.12E-05	<1.0E-07	0.723	0.999
REGA-3G12	Hu MMP-9 catalytic domain	3.1	0.318	9.74E-11	<1.0E-12	2.18E+05	2.94E+02	2.12E-05	<1.0E-07	0.723	0.999
REGA-3G12	Hu MMP-9 catalytic domain	1.6	0.194	9.74E-11	<1.0E-12	2.18E+05	2.94E+02	2.12E-05	<1.0E-07	0.723	0.999
REGA-3G12	Hu MMP-9 catalytic domain	0.8	0.115	9.74E-11	<1.0E-12	2.18E+05	2.94E+02	2.12E-05	<1.0E-07	0.723	0.999

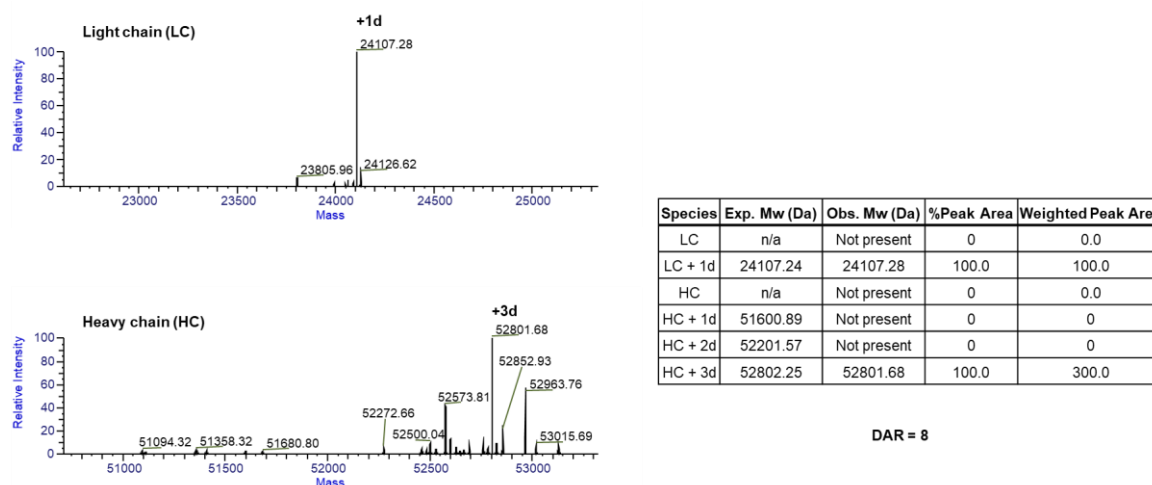
**Figure S3. Bio-Layer Interferometry (BLI) curves and fitting curves with full kinetic data obtained for REGA-3G12 binding to human MMP-9 catalytic domain.**



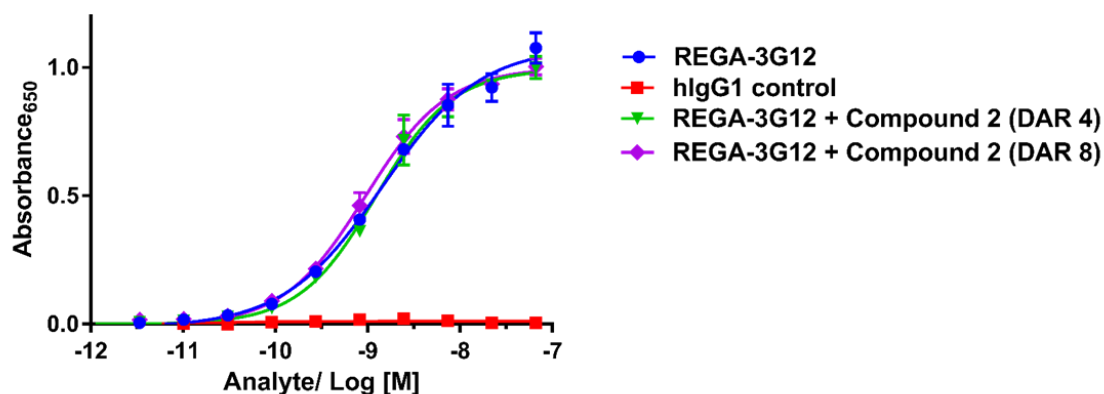
**Figure S4. REGA-3G12 does not bind to human MMP-2 ELISA against human MMP-2. Inset: anti-MMP-2 antibody MAB902 (R&D Systems) was used as a positive control in all ELISAs against MMP-2. Included on a separate scale as requires a different secondary antibody (anti-Mouse Fc-HRP, Sigma Aldrich).**



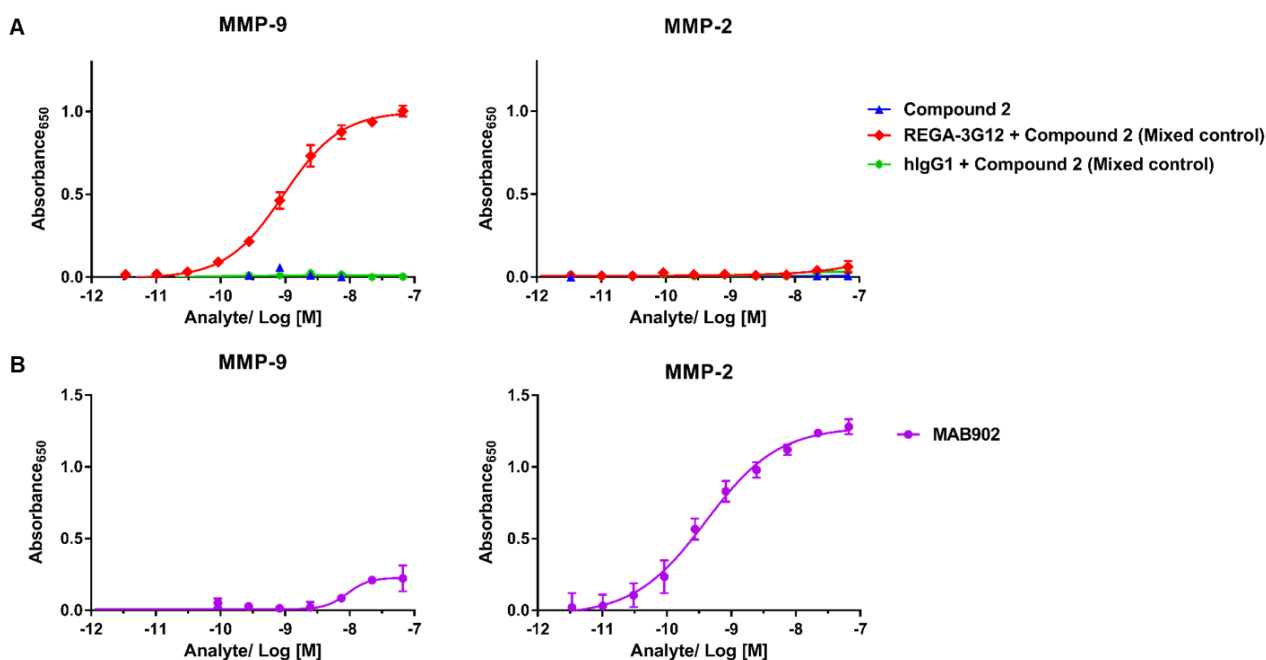
**Figure S5. Mass spectrometry analysis after incubating reduced REGA-3G12 with 4.4 equivalents of Compound 2 (referred to as 'd') to generate ADC.** Antibody was reduced with 50 mM DTT at 37 °C for 30 min prior to analysis. The relative intensities of the major mass peaks were compared and used to calculate the weighted average drug-to-antibody ratio (DAR).



**Figure S6. Mass spectrometry analysis after incubating reduced REGA-3G12 with 8.8 equivalents of Compound 2 (referred to as 'd') to generate ADC.** Antibody was reduced with 50 mM DTT at 37 °C for 30 min prior to analysis. The relative intensities of the major mass peaks were compared and used to calculate the weighted average drug-to-antibody ratio (DAR).



**Figure S7.** ELISA against human catalytic MMP-9 demonstrating REGA-3G12 maintains binding after conjugation to compound 2.



**Figure S8. Selectivity ELISA controls.** A) Compound 2 alone, and REGA-3G12 or isotype control hlgG1 mixed with 2 (no conjugation) were tested for any cross-reactivity in the ELISA against human catalytic MMP-9 and human MMP-2. B) MAB902 (R&D Systems) was used as a positive control for MMP-2 in ELISAs. Included on a separate scale as requires a different secondary antibody (anti-Mouse Fc-HRP, Sigma Aldrich).

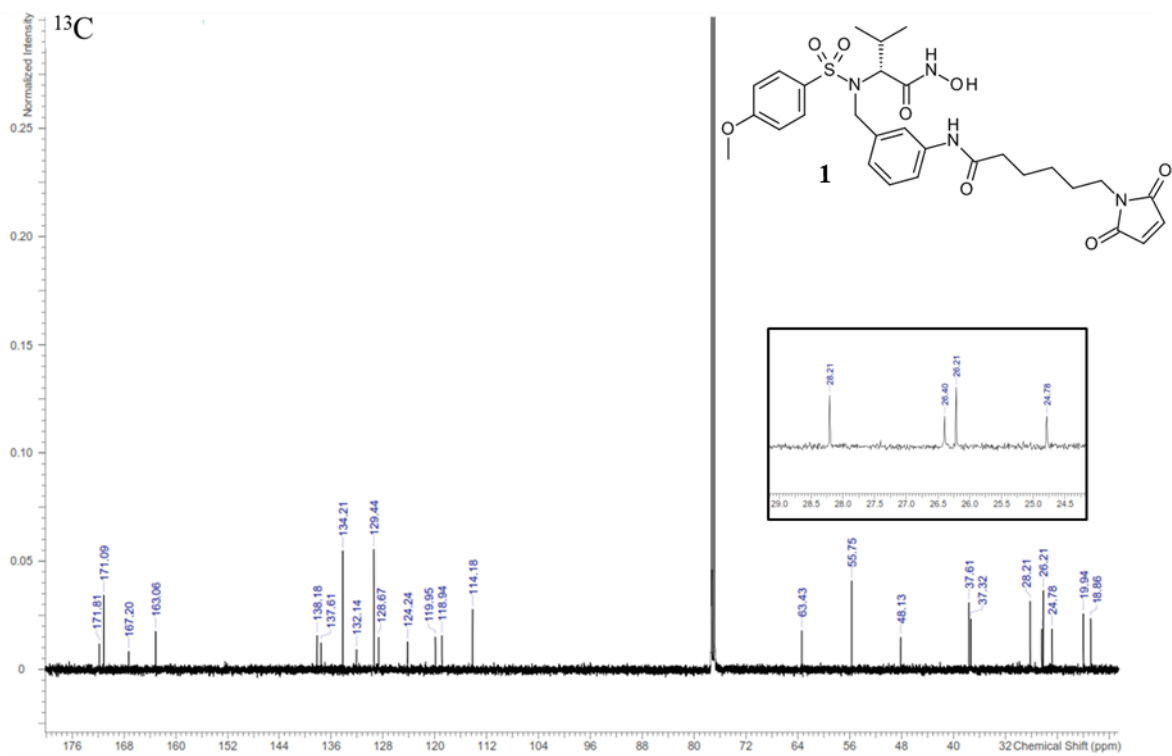
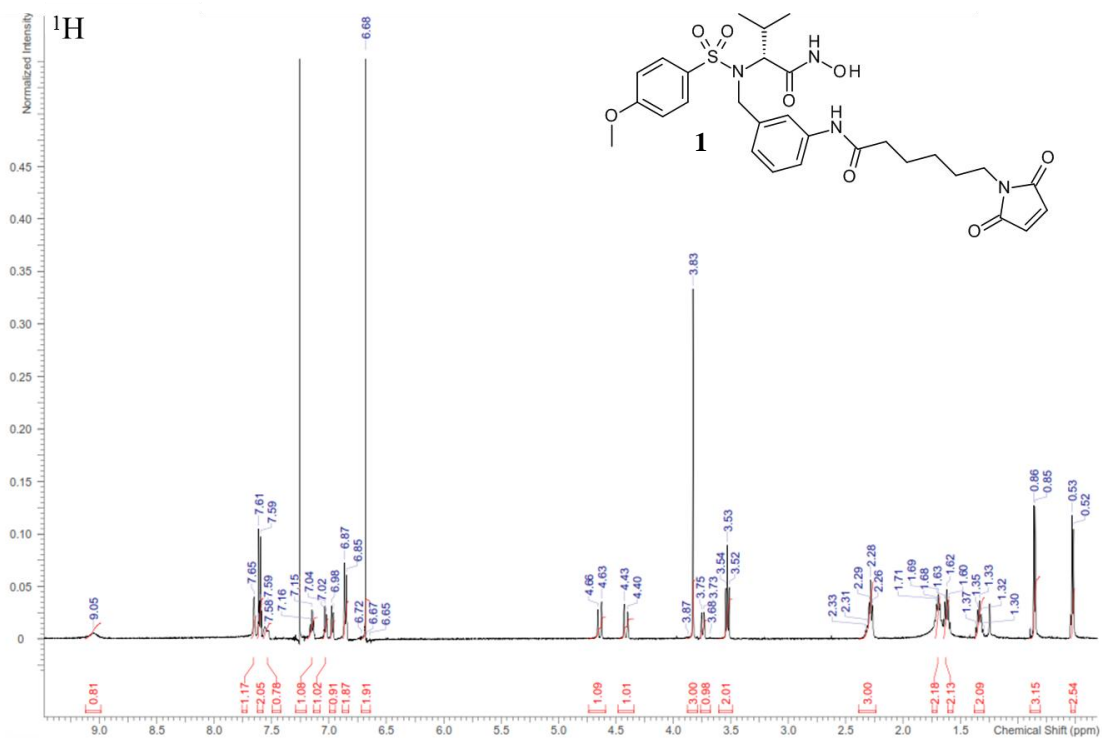
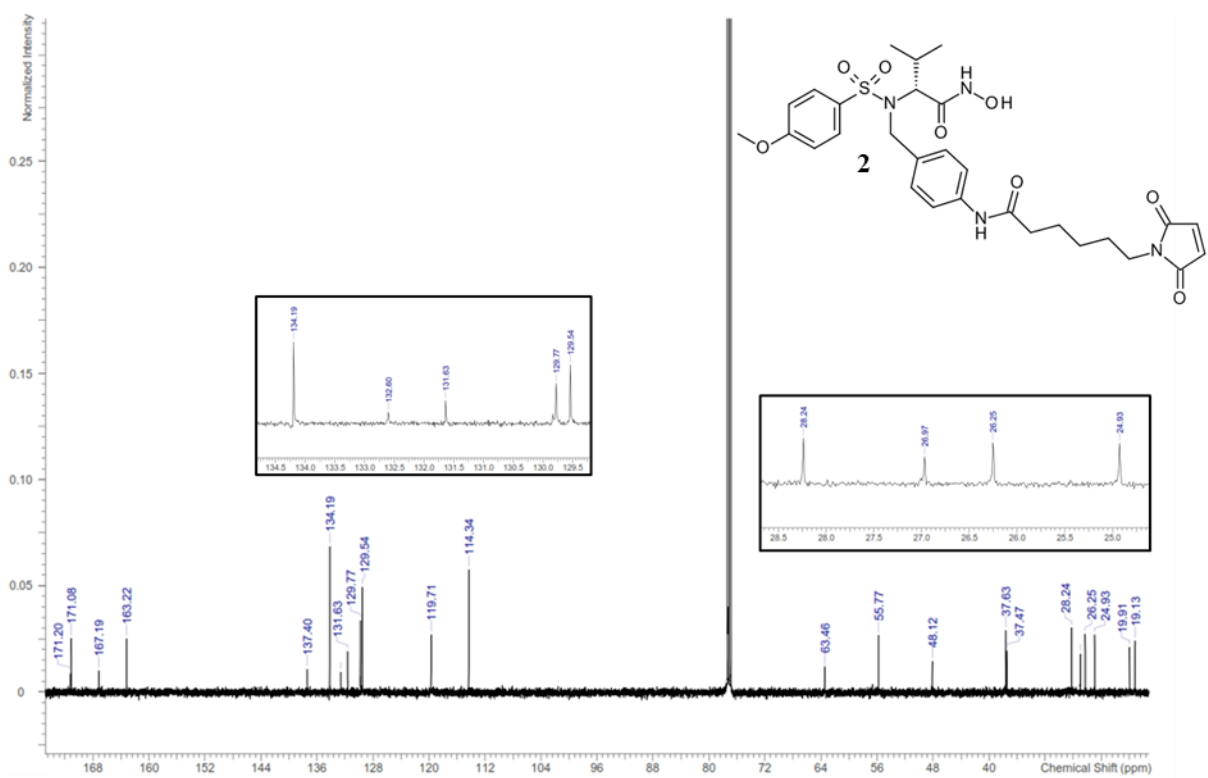
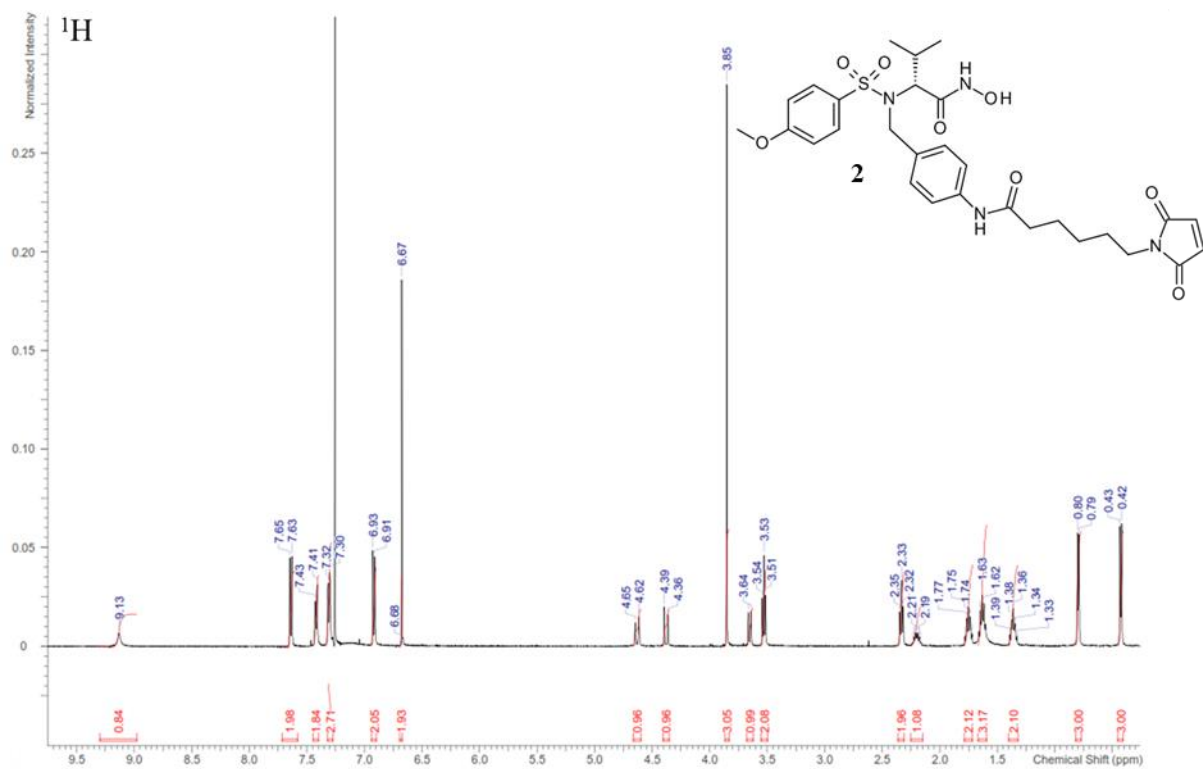


Figure S9. NMR spectra of CGS27023A-linker derivative **1**.





**Figure S10. NMR spectra of CGS27023A-linker derivative 2.**

## Supplementary Methods

### Chemical Synthesis

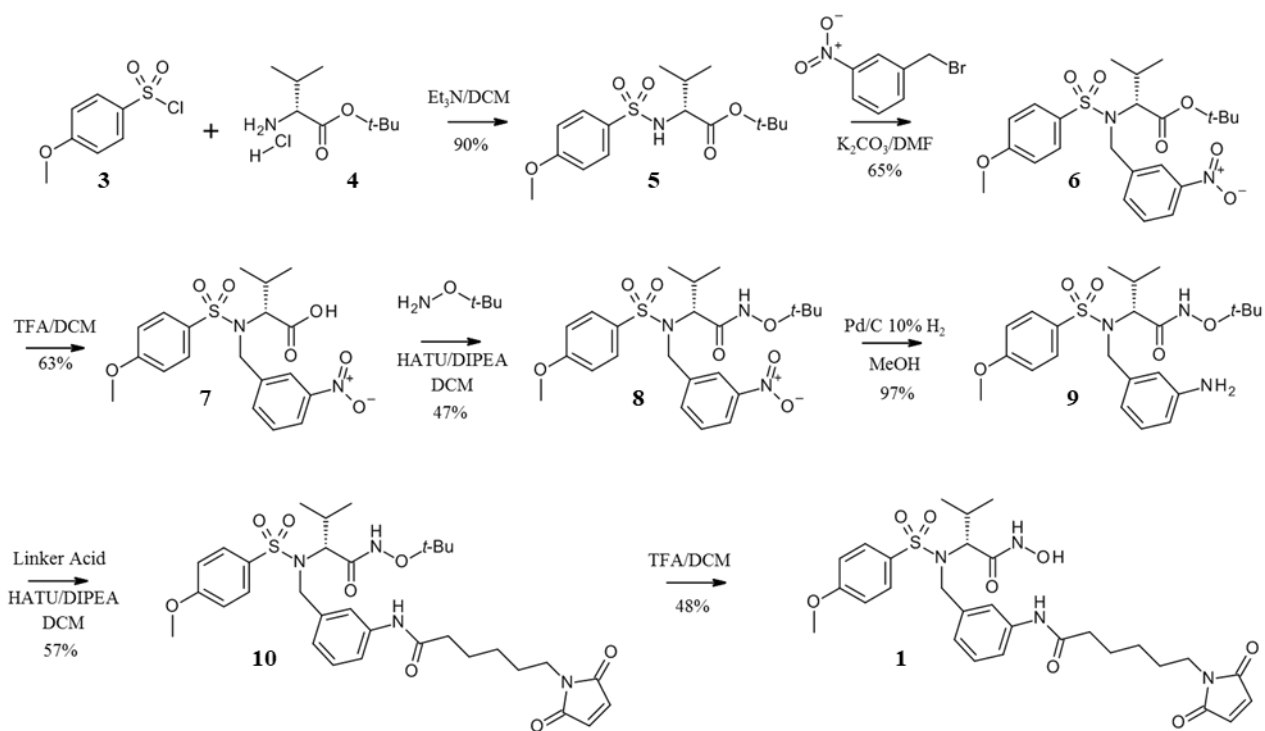
Unless otherwise specified, all reagents and solvents were purchased from Sigma-Aldrich, Fluorochem and other major suppliers and used without further purification. All reactions were monitored by thin-layer chromatography (TLC) on pre-coated TLC plates ADAMANT UV<sub>254</sub> silica gel 60 with fluorescent indicator UV<sub>254</sub>. Flash column chromatography was carried out with Biotage KP-Sil Snap cartridges using a Biotage Isolera-One.

LC-MS was carried out on an Agilent 6120 quadrupole LC-MS with an Xbridge C18 column (3.5  $\mu\text{m}$  particle and 4.6 x 30 mm dimension) and a diode array UV detector. Flow rate: 3 ml/min; Run time: 3.20 min. Solvent systems: For pH 1; Solvent A: 0.1% TFA in water, Solvent B: Acetonitrile; Gradient - 10-100% Acetonitrile; Gradient time: 2.35 min. For pH 10; Solvent A: 0.1% ammonia in water, Solvent B: Acetonitrile; Gradient - 10-100% Acetonitrile; Gradient time: 2.35 min.

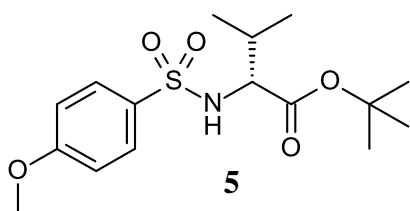
Purification of compounds by HPLC prep was carried out on an Agilent Reverse Phase Mass Directed Prep HPLC system by a member of the purification team. Purification of compounds by column chromatography were carried out on a Biotage Isolera with prepacked columns of silica.

NMR solvents were obtained from Cambridge Isotope Labs. All <sup>1</sup>H NMR (500 MHz) spectra were recorded on a JEOL ECA-500 spectrometer at 21 °C unless otherwise stated. Chemical shifts ( $\delta$ ) are in ppm and are relative to residual undeuterated NMR solvent. Coupling constants ( $J$ ) are recorded in Hertz (Hz) to the nearest 0.1 ppm.

High Resolution Mass Spectra were recorded on a Thermo-Fisher Q-Exactive operating at 70,000 Resolution.

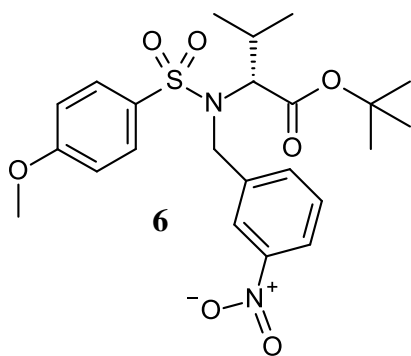


**Scheme S1. Synthesis of CGS27023A-linker derivative 1.**



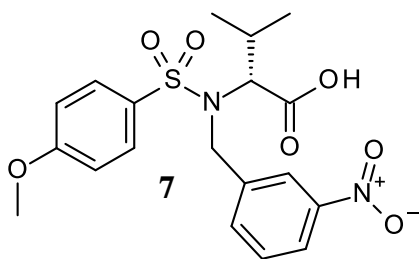
Amine **4** (1.00 g, 4.77 mmol) was stirred in DCM (30 ml) and triethylamine (1.01 g, 10.0 mmol) added. Sulfonyl chloride **3** (0.99 g, 4.77 mmol) in DCM (10 ml) was then added dropwise. The reaction mixture was stirred at rt for 6 h, then left to stand overnight. H<sub>2</sub>O was added and the reaction mixture extracted into DCM. The combined organic layers were washed with brine, dried over MgSO<sub>4</sub>, filtered and the solvent removed *in vacuo*. Purification by flash column chromatography on silica gel (5-30% EtOAc in petrol) gave desired product **5** as a white solid (1.47 g, 90%).

$^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.60-7.84 (m, 2H), 6.88-7.03 (m, 2H), 5.04 (d,  $J=10.07$  Hz, 1H), 3.83 (s, 3H), 3.52-3.63 (m, 1H), 1.94-2.11 (m, 1H), 1.22 (s, 9H), 0.98 (d,  $J=6.87$  Hz, 3H), 0.82 (d,  $J=6.87$  Hz, 3H); No mass ion observed.



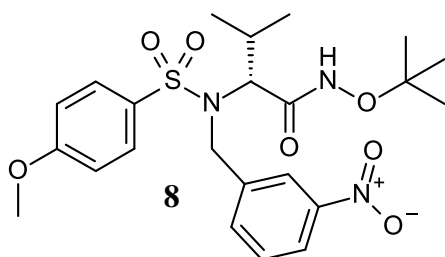
Compound **5** (790 mg, 2.30 mmol) and 1-(bromomethyl)-3-nitro-benzene (545 mg, 2.53 mmol) were stirred in DMF (30 ml).  $\text{K}_2\text{CO}_3$  (3.18 g, 23.0 mmol) was added portion-wise and the reaction mixture stirred at rt overnight.  $\text{H}_2\text{O}$  was added and the reaction mixture extracted into EtOAc. The combined EtOAc layers were washed with brine, dried over  $\text{MgSO}_4$ , filtered and the solvent removed *in vacuo* to give crude product. Purification by flash column chromatography on silica gel (5-15% EtOAc in petrol) gave desired the product **6** (715 mg, 65%).

$^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.97-8.10 (m, 2H), 7.79 (d,  $J=7.79$  Hz, 1H), 7.52-7.61 (m, 2H), 7.43 (t,  $J=8.01$  Hz, 1H), 6.73-6.87 (m, 2H), 4.62-4.82 (m, 2H), 4.16 (d,  $J=10.53$  Hz, 1H), 3.81 (s, 3H), 1.81-1.98 (m, 1H), 1.33-1.39 (m, 9H), 0.90 (d,  $J=3.21$  Hz, 3H), 0.88 (d,  $J=3.21$  Hz, 3H); No mass ion observed.



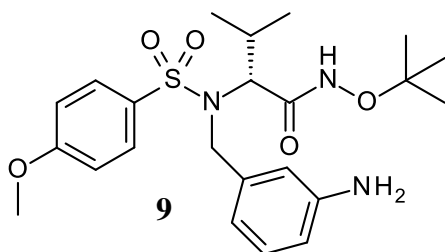
Compound **6** (715 mg, 1.69 mmol) was stirred in TFA (5 ml) overnight and then concentrated *in vacuo* to give the desired product **7** (675 mg, 63%).

$^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  8.05-8.10 (m, 1H), 8.02-8.05 (m, 1H), 7.79 (d,  $J=8.24$  Hz, 1H), 7.62-7.68 (m, 2H), 7.47 (t,  $J=8.01$  Hz, 1H), 6.84-6.91 (m, 2H), 4.56-4.76 (m, 2H), 4.25 (d,  $J=10.53$  Hz, 1H), 3.83 (s, 3H), 1.91-2.03 (m, 1H), 0.94 (d,  $J=6.41$  Hz, 3H), 0.83 (d,  $J=6.41$  Hz, 3H); No mass ion observed.



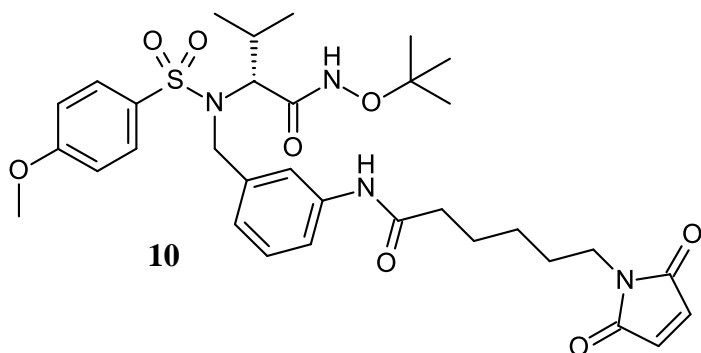
Compound **7** (675 mg, 1.60 mmol) and *O*-*tert*-butylhydroxylamine (200 mg, 1.59 mmol) were stirred in DMF (15 ml). 1-hydroxybenzotriazole (216 mg, 1.60 mmol), *N*-methylmorpholine (808 mg, 7.99 mmol) and EDC.HCl (399 mg, 2.08 mmol) were then added and the reaction mixture stirred overnight at rt. After which,  $\text{H}_2\text{O}$  was added and the reaction mixture extracted into EtOAc (3x). The combined EtOAc layers were washed with  $\text{H}_2\text{O}$ , brine, dried over  $\text{MgSO}_4$ , filtered and the solvent removed *in vacuo* to give crude product. Purification by flash column chromatography on silica gel (10-40% EtOAc in petrol) gave desired product **8** (368 mg, 47%).

$^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  8.28-8.51 (m, 1H), 7.92-8.17 (m, 2H), 7.69 (d,  $J=7.33$  Hz, 1H), 7.50-7.58 (m, 2H), 7.34-7.43 (m, 1H), 6.75-6.87 (m, 2H), 4.59-4.72 (m, 2H), 3.71-3.88 (m, 4H), 2.04-2.17 (m, 1H), 1.22 (m, 9H), 0.86 (d,  $J=6.41$  Hz, 2H), 0.62 (d,  $J=6.87$  Hz, 3H); LRMS calculated for  $\text{C}_{23}\text{H}_{32}\text{N}_3\text{O}_7\text{S}$   $[\text{MH}]^+$  494, observed 494.



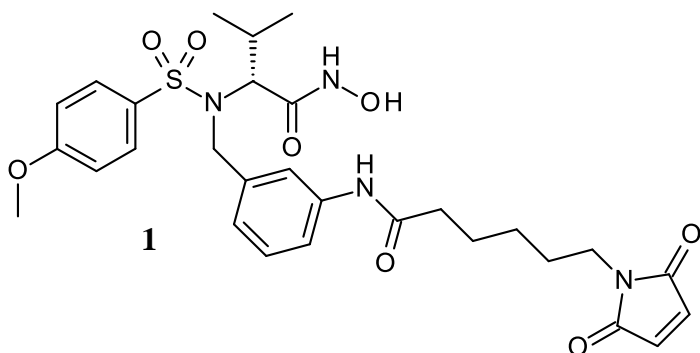
Compound **8** (368 mg, 0.75 mmol) was dissolved in MeOH (40 ml) and passed through the H-cube fitted with a 10% Pd/C Catcart at 1 ml/min, rt, full  $\text{H}_2$  mode. The reaction mixture was concentrated *in vacuo* to give desired product **9** (335 mg, 97%).

$^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  8.40 (s, 1H), 7.47-7.67 (m, 2H), 6.96-7.03 (m, 1H), 6.83-6.88 (m, 2H), 6.73-6.76 (m, 2H), 6.59 (dd,  $J=0.92, 7.79$  Hz, 1H), 4.34-4.60 (m, 2H), 3.80 (s, 3H), 3.62-3.71 (br s, 2H), 2.06-2.22 (m, 1H), 1.10-1.28 (m, 9H), 0.81 (d,  $J=6.41$  Hz, 3H), 0.54 (br d,  $J=6.87$  Hz, 3H); LRMS calculated for  $\text{C}_{23}\text{H}_{33}\text{N}_3\text{O}_5\text{S}$   $[\text{MH}]^+$  464, observed 464.



Compound **9** (120 mg, 0.26 mmol) and 6-(2,5-dioxopyrrol-1-yl)hexanoic acid (55 mg, 0.26 mmol) were dissolved in DCM (20 ml). HATU (108 mg, 0.28 mmol) was added followed by DIPEA (100 mg, 0.77 mmol), and the reaction mixture stirred overnight at rt. H<sub>2</sub>O (10 ml) was added and the DCM layer isolated using a phase separator cartridge and then concentrated *in vacuo* to give crude product. Purification by flash column chromatography on silica gel (10-50% EtOAc in Petrol) gave desired product **10** (97 mg, 57%).

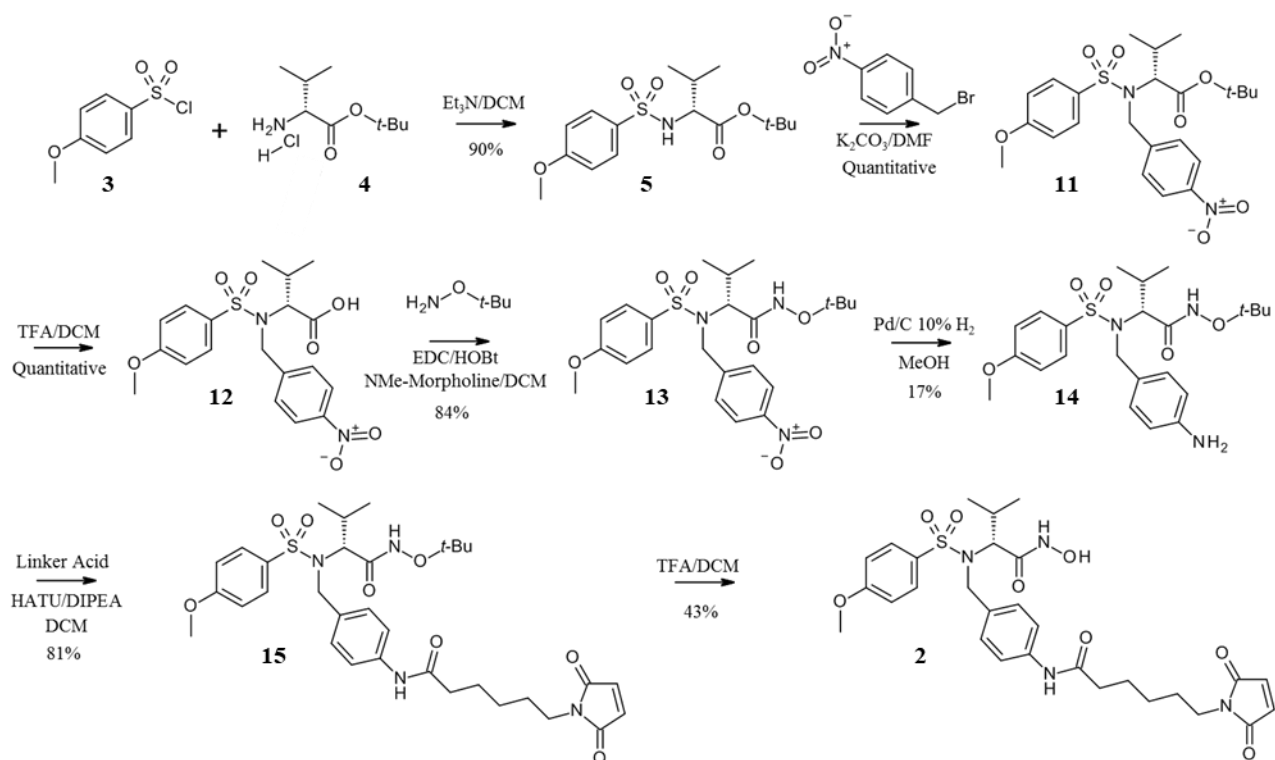
<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.41 (s, 1H), 7.66 (br d, *J*=8.70 Hz, 1H), 7.55-7.59 (m, 2H), 7.53 (s, 1H), 7.22 (s, 1H), 7.16 (t, *J*=7.79 Hz, 1H), 7.04 (br d, *J*=7.33 Hz, 1H), 6.83-6.88 (m, 2H), 6.66 (s, 2H), 4.40-4.59 (m, 2H), 3.81 (s, 3H), 3.67 (d, *J*=10.99 Hz, 1H), 3.51 (t, *J*=7.33 Hz, 2H), 2.30 (t, *J*=7.33 Hz, 2H), 2.08-2.21 (m, 1H), 1.72 (m, 2H), 1.61 (m, 2H), 1.30-1.39 (m, 2H), 1.22 (s, 9H), 0.83 (d, *J*=6.41 Hz, 3H), 0.52 (d, *J*=6.41 Hz, 3H); LRMS calculated for C<sub>33</sub>H<sub>44</sub>N<sub>4</sub>O<sub>8</sub>S [MH]<sup>+</sup> 657, observed [MH]<sup>+</sup> 657.



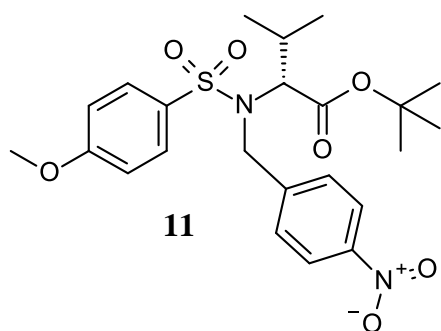
To a stirred solution of **10** (97 mg, 0.15 mmol) in DCM (5 ml) was added TFA (3 ml, 39.68 mmol). The reaction mixture was stirred at rt for 5 days, after which the solution was concentrated *in vacuo*. Purification by reverse phase column chromatography on a Biotage Ultra C18 12 g snap cartridge eluting with MeOH:0.1%TFA in H<sub>2</sub>O 20-80% gave desired product **1** as a white solid (43 mg, 48%).

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.63 (br s, 1H), 7.57-7.61 (m, 4H), 7.09-7.17 (m, 1H), 7.03 (br d, *J*=8.24 Hz, 1H), 6.96 (d, *J*=7.33 Hz, 1H), 6.81-6.86 (m, 2H), 6.67 (s, 2H), 4.63 (d, *J*=16.03 Hz, 1H), 4.41 (d, *J*=16.03 Hz, 1H), 3.80-3.83 (s, 3H), 3.72-3.78 (m, 1H), 3.52 (t, *J*=7.10 Hz, 2H), 2.23-2.35 (m, 3H), 1.55-1.73 (m, 4H), 1.32 (m, 2H), 0.84 (d, *J*=5.95 Hz, 3H), 0.52 (d, *J*=6.41 Hz, 3H); <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>) δ 171.81, 171.09, 167.20, 163.06, 138.18, 137.61, 134.21, 132.14, 129.44, 128.67, 124.24, 119.95, 118.94, 114.18, 63.43, 55.75, 48.13, 37.61, 37.32, 28.21, 26.40, 26.21, 24.78, 19.94, 18.86; HRMS calculated for C<sub>29</sub>H<sub>37</sub>N<sub>4</sub>O<sub>8</sub>S [MH]<sup>+</sup> 601.2332, observed 601.2323.





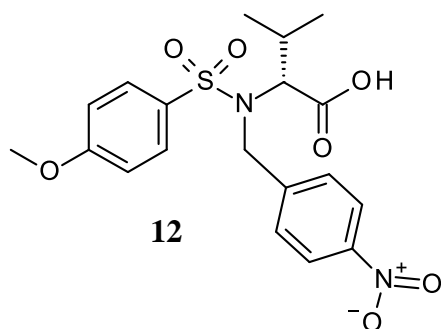
### Scheme S2. Synthesis of CGS27023A-linker derivative 2



To a stirred solution of **5** (3.0 g, 8.74 mmol) in DMF (30 ml),  $K_2CO_3$  (12 g, 87.0 mmol) was added portion-wise, followed by the portion-wise addition of 1-(bromomethyl)-4-nitrobenzene (1.98 g, 9.26 mmol). The reaction mixture was stirred at rt for 4 h, following which  $H_2O$  was added and the reaction mixture extracted into EtOAc. The combined EtOAc layers were washed with  $H_2O$ , brine, dried over  $MgSO_4$ , filtered and the solvent removed *in vacuo* to give desired product **11** (4.4 g, quantitative).

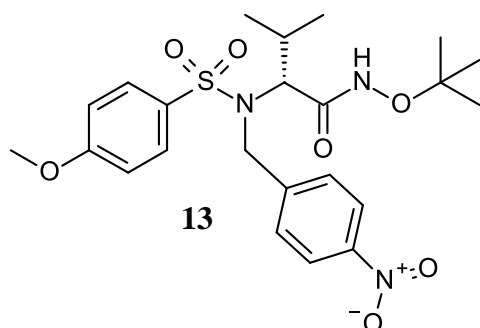
$^1H$  NMR (500 MHz,  $CDCl_3$ )  $\delta$  8.08-8.12 (m, 2H), 7.63-7.67 (m, 2H), 7.56-7.60 (m, 2H), 6.86-

6.90 (m, 2H), 4.78 (d,  $J=16.61$  Hz, 1H), 4.69 (d,  $J=16.04$  Hz, 1H), 4.09-4.13 (m, 1H), 3.84 (s, 3H), 1.78-1.86 (m, 1H), 1.32 (s, 9H), 0.86 (d,  $J=6.87$  Hz, 3H), 0.83 (d,  $J=6.87$  Hz, 3H); No mass ion observed.



Compound **11** (4.4 g, 9.19 mmol) was stirred in TFA (10 ml, 132 mmol) at rt for 2 h. The reaction mixture was then concentrated *in vacuo* to give the desired product **12** (4.2 g, quantitative).

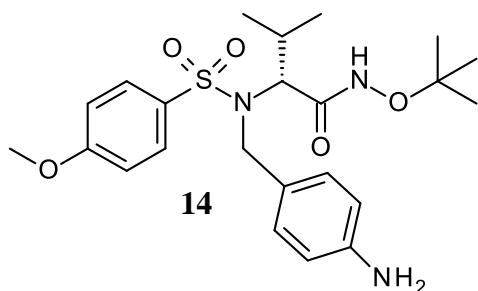
$^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  8.11-8.15 (m, 2H), 7.68-7.72 (m, 2H), 7.55-7.59 (m, 2H), 6.89-6.94 (m, 2H), 4.71 (d,  $J=16.61$  Hz, 1H), 4.64 (d,  $J=16.61$  Hz, 1H), 4.19 (d,  $J=10.31$  Hz, 2H), 3.85 (s, 3H), 1.85-1.95 (m, 1H), 0.91 (d,  $J=6.87$  Hz, 3H), 0.78 (d,  $J=6.87$  Hz, 3H); No mass ion observed.



To a stirred solution of **12** (4.2 g, 9.94 mmol) in DMF (20 ml) was added 4-methylmorpholine (5.05 g, 49.4 mmol), 1-hydroxybenzotriazole (1.3 g, 9.62 mmol) and EDC.HCl (2.0 g, 13.0

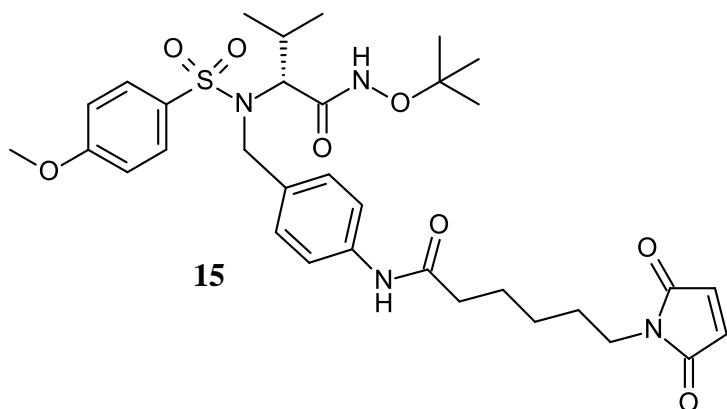
mmol). The reaction mixture was stirred overnight at rt, after which H<sub>2</sub>O was added and the reaction mixture extracted into EtOAc. The combined EtOAc layers were washed with H<sub>2</sub>O, brine, dried over MgSO<sub>4</sub>, filtered and the solvent removed *in vacuo* to give desired product **13** (4.1 g, 84%).

<sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.06-8.20 (m, 3H), 7.58-7.63 (m, 2H), 7.49-7.54 (m, 2H), 7.52 (d, *J*=8.59 Hz, 2H), 6.87-6.94 (m, 2H), 4.58-4.72 (m, 2H), 3.79-3.91 (m, 3H), 3.67 (d, *J*=10.88 Hz, 1H), 2.05-2.12 (m, 1H), 1.18-1.25 (m, 9H), 0.86 (d, *J*=6.30 Hz, 3H), 0.55 (d, *J*=6.30 Hz, 3H); LRMS calculated for C<sub>23</sub>H<sub>31</sub>N<sub>3</sub>O<sub>7</sub>S [MH]<sup>+</sup> 494, observed 494.



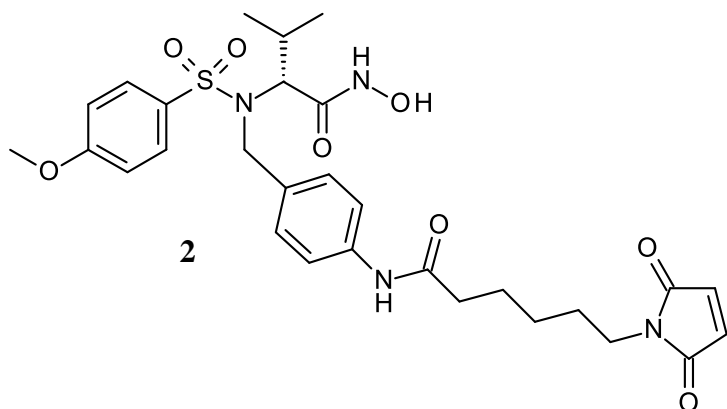
Compound **13** (4.1 g, 8.31 mmol) was dissolved in methanol (300 ml) and the reaction mixture passed through the H-Cube fitted with a 10% Pd/C CatCart at 1 ml/min, rt, full H<sub>2</sub>. Fractions were collected and analysed. The pure fractions were combined and concentrated *in vacuo* to give desired product **14** as a white solid (650 mg, 17% yield). Other impure fractions were combined to give an orange solid (2.72 g).

<sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.27 (s, 1H), 7.51-7.58 (m, 2H), 7.11-7.17 (m, 2H), 6.82-6.90 (m, 2H), 6.49-6.58 (m, 2H), 4.55 (d, *J*=14.89 Hz, 1H), 4.36 (d, *J*=14.89 Hz, 1H), 3.79-3.87 (m, 3H), 3.62 (d, *J*=11.46 Hz, 1H), 2.15-2.22 (m, 1H), 1.25 (s, 9H), 0.84 (d, *J*=6.30 Hz, 3H), 0.48 (d, *J*=6.87 Hz, 3H); No mass ion observed.



To a stirred solution of **14** (650 mg, 1.40 mmol) in DCM (40 ml) was added 6-(2,5-dioxopyrrol-1-yl)hexanoic acid (315 mg, 1.40 mmol), HATU (586 mg, 1.54 mmol) and DIPEA (538 mg, 4.21 mmol). The reaction mixture was stirred at room temp for 3 h, after which H<sub>2</sub>O was added and the reaction mix stirred for 10 min. The reaction mixture was then passed through a phase separator cartridge and the DCM layer concentrated *in vacuo*. Purification by flash column chromatography on silica gel (20-80% EtOAc in Petrol) gave the desired product **15** in good yield (748 mg, 81%).

<sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.21 (s, 1H), 7.55-7.61 (m, 2H), 7.37-7.42 (m, 2H), 7.27-7.32 (m, 2H), 7.19-7.23 (m, 1H), 6.86-6.93 (m, 2H), 6.67 (s, 2H), 4.60 (d, *J*=15.47 Hz, 1H), 4.47 (d, *J*=16.04 Hz, 1H), 3.84 (s, 3H), 3.63 (d, *J*=10.88 Hz, 1H), 3.53 (t, *J*=7.16 Hz, 2H), 2.33 (t, *J*=7.45 Hz, 2H), 2.09-2.22 (m, 1H), 1.75 (quin, *J*=7.59 Hz, 1H), 1.71-1.83 (m, 1H), 1.61-1.68 (m, 2H), 1.31-1.43 (m, 2H), 1.25 (s, 9H), 0.83 (d, *J*=6.87 Hz, 3H), 0.47 (d, *J*=6.87 Hz, 3H); LRMS calculated for C<sub>33</sub>H<sub>44</sub>N<sub>4</sub>O<sub>8</sub>S [MH]<sup>+</sup> 657, observed 657.



To a stirred solution of **15** (740 mg, 1.13 mmol) in DCM (35 ml) was added TFA (10 ml, 132 mmol). The reaction mixture was stirred at rt for 1 week, and then concentrated *in vacuo*. The crude product was purified by reverse phase column chromatography on a 12 g Biotage Ultra C18 Snap cartridge eluting with MeCN:0.1%TFA in H<sub>2</sub>O 10-95%. The fractions containing the desired product were concentrated *in vacuo* and then dried overnight in the drying pistol under vacuum to yield the desired product **2** as a white solid (0.29 g, 43%).

<sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 9.13 (br s, 1H), 7.64 (d, *J*=8.59 Hz, 2H), 7.42 (br d, *J*=8.02 Hz, 2H), 7.31 (br d, *J*=8.59 Hz, 3H), 6.92 (d, *J*=8.59 Hz, 2H), 6.67 (s, 2H), 4.63 (br d, *J*=15.47 Hz, 1H), 4.38 (d, *J*=15.47 Hz, 1H), 3.85 (s, 3H), 3.65 (d, *J*=10.88 Hz, 1H), 3.53 (t, *J*=7.16 Hz, 2H), 2.33 (t, *J*=7.45 Hz, 2H), 2.14-2.26 (m, 1H), 1.75 (quin, *J*=7.59 Hz, 2H), 1.63 (quin, *J*=7.30 Hz, 3H), 1.36 (quin, *J*=7.59 Hz, 2H), 0.80 (d, *J*=6.30 Hz, 3H), 0.43 (d, *J*=6.87 Hz, 3H); <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>) δ 171.20, 171.08, 167.19, 163.22, 137.40, 134.19, 132.60, 131.63, 129.83, 129.54, 119.71, 114.34, 63.52, 55.77, 48.12, 37.63, 37.47, 28.24, 26.97, 26.25, 24.93, 19.91, 19.13; HRMS calculated for C<sub>29</sub>H<sub>37</sub>N<sub>4</sub>O<sub>8</sub>S [MH]<sup>+</sup> 601.2332, observed 601.2323.

## **REGA-3G12 Expression and Purification**

REGA-3G12 heavy and light chain DNA were assembled by inserting variable regions into in house expression vectors containing antibody constant regions by LIC cloning. The antibody was expressed by transient transfection in Expi293F<sup>TM</sup> cells following the Expifectamine<sup>TM</sup> 293 transfection kit protocol provided by Life Technologies. Heavy and light chain plasmid DNA were used in a 1:1 ratio at a total concentration of 1 µg plasmid DNA per ml culture. The expression medium containing the secreted antibody was harvested 7 days after transfection. REGA-3G12 was purified by standard Protein A chromatography, followed by size exclusion (HiLoad 16/600 Superdex 200 pg, GE Healthcare). The purified antibody was characterized by SDS-PAGE and LC-MS (Thermo Scientific Q-Exactive<sup>TM</sup> BioPharma platform).

## **REGA-3G12 Sequence**

### *Heavy chain*

QVQLQQSGAELVMPGASVKMSCASGYTFTDYWMHWVKQRPGQGLEWIGAITSDTYTRYNQKFKGKATLTVDESSSTAYMQASSLTSEDSAVYYCARAVIIYGSSWGYFDVWGQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK

### *Light chain*

DIELTQSPSYLAASPGETITINCRASKSISKYLAWYQEKP GKTNKLLIYSGSTLQSGIPSRFSGSGSGTDFTLTISSLEPEDFAMYYCQQHNEYPTFGGGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEEKHKVYACEVTHQGLSSPVTKSFNRGEC

## **Antibody LC-MS analysis**

LC-MS was performed on a Thermo Scientific Q-Exactive Plus Orbitrap mass spectrometer coupled to Vanquish Flex UHPLC system using an Agilent PLRP-S column (1,000 Å, 5 µm,

2.1 mm x 50 mm). Flow rate: 0.250 ml/min; Run time: 6 min. Solvent A: 0.1% formic acid in water, Solvent B: Acetonitrile; Gradient: 15-90%. Column temperature: 80 °C. All mass spectrometry results were analysed using Thermo Scientific BioPharma Finder informatics platform for protein characterization.

### **Antibody Conjugation and Purification**

Protocol adapted from Puthenveetil *et al*<sup>1</sup>. All conjugation steps carried out at room temperature with end-over-end mixing.

To conjugate, a NAb™ Protein A Spin Column, 0.2 ml (ThermoScientific) was equilibrated by washing with 400 µl PBS three times. The column was capped and antibody in PBS added. After 30 mins, 200 equiv of TCEP was added and the antibody reduced for 1 h. The antibody bound beads were then washed three times with 400 µl PBS + 5 mM EDTA (pH 7.0). Either 4.4 or 8.8 equiv of small molecule was subsequently added in 400 µl PBS + 5 mM EDTA + 10% DMA (pH 7.0). After 1 h, excess small molecule was removed by washing the column with 400 µl PBS + 10% DMA (pH 7.0) five times. Following the washes, the ADC was released from the column using 400 µl 100 mM glycine.HCl buffer (pH 3.0) eluting into 40 µl of neutralizing 1 M Tris buffer (pH 8.0), this was repeated a further two times. The resulting three ADC fractions were combined and buffer exchanged into PBS.

### **proMMP-2 Expression and Purification**

The proMMP-2 gene with c-terminal Avitag was inserted into pcDNA3.1 (+) mammalian expression vector by a commercial provider. The metalloproteinase was expressed by transient transfection in Expi293F™ cells following the Expifectamine™ 293 transfection kit protocol provided by Life Technologies. 1 µg plasmid DNA was used per ml culture. The expression

medium containing the secreted protein was harvested 7 days after transfection. proMMP-2 was purified using Gelatin Sepharose 4B affinity resin (GE Healthcare) followed by size exclusion (HiLoad 16/600 Superdex 200 pg, GE Healthcare). The purified metalloproteinase was characterized by SDS-PAGE and activity confirmed by the MMP fluorometric assay.

### **proMMP-2 Sequence**

MEALMARGALTGPLRALCLLGCLLSHAAAAPSPIIKFPGDVAPKTDKELAVQYLNTF  
YGCPKESC�LFVLKDTLKKMQFFGLPQTGDLQNTIETMRKPRCGNPDVANYNFF  
PRKPKWDKNQITYRIIGYTPDLDPETVDDAFARAFQVWSDVTPLRFSRIHDGEADIMI  
NFRWEHGDGYPFDGKDGLLAHAFAPGTGVGGDSHFDDDELWTLGEGQVVRVKY  
GNADGEYCKFPFLFNGKEYNSCTDTGRSDGFLWCSTTYNFEKDGGKYGFCPHEALFT  
MGGNAEGQPCKFPFRFQGTSYDSCTTEGRTDGYRWCGTTEDYDRDKKYGFCPETA  
MSTVGGNSEGAPCVFPFTFLGNKYESCTSAGRSDGKMWCATTANYDDDRKWGFCP  
DQGYSLFLVAAHEFGHAMGLEHSQDPGALMAPIYTYTKNFRLSQDDIKGIQELYGAS  
PDIDLGTGPTPTLGPVTPEICKQDIVFDGIAQIRGEIFFFKDRFIWRTVTPRDKPMGPLL  
VATFWPELPEKIDAVYEAPQEEKAVFFAGNEYWIYSASTLERGYPKPLTSLGLPPDV  
QRVDAAFNWSKNKTYIFAGDKFWRYNEVKKKMDPGFPKLIADAWNAIPDNLDAV  
VDLQGGGHSYFFKGAYYLKLENQSLKSVKFGSIKSDWLGCCGSGGLNDIFEAQKIE  
WHE

### **proMMP-2 Activation with APMA**

proMMP-2 was activated by addition of 1 mM APMA (AnaSpec) for 1 h at 37 °C immediately before use in BLI, ELISA or fluorometric assays.

### **MMP-9 ELISA**

ELISA plates were coated with human MMP-9 catalytic domain (AnaSpec) diluted to a final concentration of 1 µg/ml in PBS and incubated overnight at 4 °C. The plate was emptied of liquid and then blocked for 1 h at room temperature with 2% BSA in PBST (PBS + 0.05% Tween-20). Plates were washed three times with PBS + 0.1% Tween-20, and the serially diluted test samples (66.6 nM, 22.2 nM, 7.41 nM, 2.47 nM, 0.82 nM, 0.27 nM, 0.09 nM, 0.03 nM, 0.01 nM, 0.003 nM) of REGA-3G12, isotype control hIgG1, REGA-3G12 + **B** (DAR 4)



and REGA-3G12 + **B** (DAR 8) were added in 0.2% BSA in PBST. The assay was incubated at room temperature for 1 h, washed three times with PBS + 0.1% Tween-20, and the secondary antibody (anti-Human Fc HRP, Sigma, 1 : 3,000 in 0.2% BSA in PBST) added. After 1 h the ELISA plates were washed again and 20  $\mu$ L enhanced K-Blue substrate (Neogen) was added. Once colour was observed, the reaction was stopped by adding 10  $\mu$ L red stop solution (Neogen). Absorbance was immediately measured at 650 nm. Controls were included in every ELISA, in which PBS had been added to some of the wells instead of MMP-9 or instead of antibody sample. Each sample was tested in triplicate, and errors are shown as the standard deviation of the average.

### **MMP-2 ELISA**

ELISA plates were coated with activated human MMP-2 (activation described above) diluted to a final concentration of 1  $\mu$ g/ml in PBS and incubated overnight at 4 °C. The plate was emptied of liquid and then blocked for 1 h at room temperature with 2% BSA in PBST (PBS + 0.05% Tween-20). Plates were washed three times with PBS + 0.1% Tween-20, and the serially diluted test samples (66.6 nM, 22.2 nM, 7.41 nM, 2.47 nM, 0.82 nM, 0.27 nM, 0.09 nM, 0.03 nM, 0.01 nM, 0.003 nM) of REGA-3G12, isotype control hIgG1 and MAB902 (R&D Systems) were added in 0.2% BSA in PBST. The assay was incubated at room temperature for 1 h, washed three times with PBS + 0.1% Tween-20, and the appropriate secondary antibody added (anti-Human Fc HRP, Sigma, 1 : 3,000 in 0.2% BSA in PBST or anti-Mouse Fc HRP, Sigma, 1 : 1000 in 0.2% BSA in PBST). After 1 h the ELISA plates were washed again and 20  $\mu$ L enhanced K-Blue substrate (Neogen) was added. Once colour was observed, the reaction was stopped by adding 10  $\mu$ L red stop solution (Neogen). Absorbance was immediately measured at 650 nm. Controls were included in every ELISA, in which PBS had been added

to some of the wells instead of MMP-2 or instead of antibody sample. Each sample was tested in triplicate, and errors are shown as the standard deviation of the average.

### **ADC selectivity ELISA**

Selectivity ELISA was run against MMP-9 and MMP-2 using the same ELISA methods as described above. The following samples were included against each metalloproteinase at the concentrations listed above: REGA-3G12, isotype control hIgG1, compound **2**, REGA-3G12 + **2** (DAR 4), hIgG1 + **2** (DAR 4), REGA-3G12 + **2** mixture (a non-conjugated DAR 4 mimic), hIgG1 + **2** mixture (a non-conjugated DAR 4 mimic), and MAB902 (R&D Systems).

### **REGA-3G12 binding kinetics (Bio-layer interferometry)**

Binding assays were performed on an Octet Red instrument (fortéBIO). All steps were conducted at 25 °C, 1,000 rpm in HBS-P+ buffer (GE Healthcare) using a 384-well tilted-bottom microplate. REGA-3G12 (1 µg/ml) was immobilized on pre-soaked (HBS-P+) Protein G sensors. A range of concentrations of either human MMP-9 catalytic domain (12.5 nM, 6.3 nM, 3.1 nM, 1.6 nM, 0.8 nM) or human MMP-2 (250 nM, 125 nM, 62.5 nM, 31.3 nM, 15.6 nM) were used to determine  $K_D$ . Association time was 900 s and dissociation time was 5,400 s. The  $K_D$  for REGA-3G12 binding to human catalytic MMP-9 was determined using fortéBio Data Analysis 9.0. Curves were fitted to 1:1 binding model.

### **MMP Fluorometric Assays**

The SensoLyte® 520 MMP-9 assay kit (AnaSpec) was used to measure activity of catalytic MMP-9 (AnaSpec) and activated full length MMP-2 (as described above) in a 384-well

microplate format according to manufacturer's instructions. Catalytic MMP-9 and full length MMP-2 were used at 1 nM final assay concentration. Inhibition assays were carried out by incubating the small molecule inhibitor, antibody or ADC with MMP-9 or MMP-2 at room temperature for 30 min. 5-FAM/QXL520™ FRET substrate was then added to the reaction mixture and the assay plate immediately read in kinetic mode at 5min intervals for 3 h on the Pherastar FS (BMG Labtech) by measuring the fluorescence intensity at excitation/emission wavelengths 490/520 nm.

#### *Data analysis*

Data was normalised to high controls at a time point on the linear phase of the kinetic read where the signal was at least 6-fold over the low controls to give a percentage activity value. Results are expressed as the mean  $\pm$  SEM from at least three independent experiments, run in duplicate. Graphs were fitted to data using GraphPad Prism (V7.03 for Windows, GraphPad Software). Concentration–response data were fitted using a four-parameter logistic equation to calculate IC<sub>50</sub> values.

#### **References**

1. Puthenveetil, S.; Musto, S.; Loganzo, F.; Tumey, L. N.; O'Donnell, C. J.; Graziani, E., Development of Solid-Phase Site-Specific Conjugation and Its Application toward Generation of Dual Labeled Antibody and Fab Drug Conjugates. *Bioconjugate chemistry* **2016**, 27 (4), 1030-9.